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Isolation and structural identification of complex feruloylated heteroxylan side-chains from maize bran

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Abstract

Three complex heteroxylan side-chains acylated with ferulate and one arabinosyl ester of *p*-coumaric acid have been isolated from maize bran insoluble fibre after acidic hydrolysis and fractionation by gel permeation chromatography and semi-preparative RP-HPLC. The complete structural elucidation of all isolated compounds was achieved by 1D/2D NMR spectroscopy and HPLC-MS in combination with methylation analysis. The absolute configuration of the carbohydrate constituents was determined by chiral GC after acidic hydrolysis and trifluoroacetylation. The identified feruloylated tetrasaccharides α-D-xylopyranosyl-(1 → 3)-α-L-galactopyranosyl-(1 → 2)-β-D-xylopyranosyl-(1 → 2)-5-*O-trans*-feruloyl-L-arabinofuranose (FAXGX) and α-D-galactopyranosyl-(1 → 3)-α-L-galactopyranosyl-(1 → 2)-β-D-xylopyranosyl-(1 → 2)-5-*O-trans*-feruloyl-L-arabinofuranose (FAXGG) are the most complex heteroxylan side-chains from maize bran that have been isolated to date. The isolated trisaccharide α-L-galactopyranosyl-(1 → 2)-β-D-xylopyranosyl-(1 → 2)-5-*O-trans*-feruloyl-L-arabinofuranose (FAXG) contributes to the complexity of heteroxylan side-chains from maize bran and 5-*O-trans-p*-coumaroyl-L-arabinofuranose represents the first *p*-coumaroylated heteroxylan side-chain isolated from cereal grains. Complex feruloylated heteroxylan side-chains are possibly, like ferulate cross-linking of the heteroxylans and binding of heteroxylans to lignin, a factor contributing to limited enzymatic degradation of fibre.

1. Introduction

Ferulic and *p*-coumaric acids are two of the major hydroxycinnamic acid derivatives, that are incorporated into plant cell walls (Ishii, 1997). Higher proportions of ferulates are located mainly in cereal brans, maize bran being an especially rich source (Bunzel et al., 2001); *p*-coumaric acid is predominant in cereal stems (Faulds and Williamson, 1999). It is well established that in most grasses ferulic acid acylates the *O*-5-position of α-L-arabinofuranosyl residues of arabinoxylans, whereas in dicots it is primarily bound to pectins (Faulds and Williamson, 1999; Ishii, 1997). The attachment of *p*-coumaric acid at the *O*-5-position of α-arabinosyl residues of arabinoxylans via an ester-bond was proven in barley straw (Mueller-Harvey et al., 1986), coastal Bermuda grass (Borneman et al., 1990), and bamboo shoots (Ishii and Hiroi, 1990; Ishii et al., 1990; Ishii, 1996).

Feruloyl residues undergo radical coupling reactions that result in cross-linking between polysaccharides, between polysaccharides and lignin (Ralph et al., 1994, 1995, 1998) and, probably, between polysaccharides and proteins (Piber and Koehler, 2005). Polysaccharide cross-linking provides structural integrity to the cell wall, limits

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the enzymatic (Grabber et al., 1998a,b) and possibly also the more complex microbial degradation of fibre, and plays an important role in plant food textural and processing properties (Parker and Waldron, 1995; Waldron et al., 1997). The influence of ferulate cross-links on polysaccharide degradation is of special interest for forage digestibility in ruminants and fermentation of dietary fibre polysaccharides in the human colon by intestinal bacteria. A range of dehydrodiferulates have been detected in various plant materials and were characterised as 8-5, 5-5-, 8-O-4-, 8-8-, and 4-O-5-coupled dehydrodimers (Bunzel et al., 2000, 2001; Parr et al., 1996, 1997; Ralph et al., 1994; Saulnier and Thibault, 1999). The isolation of 5–5-dehydrodiferuloylated saccharides (Ishii, 1991; Saulnier et al., 1999) and of an 8-O-4-dehydrodiferuloylated diarabinoside (Allerdings et al., 2005) provided the required structural evidence that dehydrodiferulates act as intra/intermolecular cross-linking agents in the cell walls. Recently, five dehydrotriferulic acids have been identified from maize bran fibre (5-5/8-O-4-, 5-5/8-O-4(H$_2$O)-, 8-O-4/8-O-4-, 8-8c/8-O-4-, and 8-O-4/8-5 (non-cyclic)-coupled dehydrotriferulic acids), indicating the possibility that more than two polysaccharide chains are cross-linked via ferulate derivatives (Bunzel et al., 2003, 2005; Funk et al., 2004).

Limited enzymatic degradation of fibre may be affected by factors additional to ferulate cross-linking, e.g. by complex feruloylated heteroxylan side-chains. Complex side-chains can, for example, decrease the access of carbohydrolases to the xylan backbone or protect the feruloyl group from enzymatic removal by feruloyl-esterases. α-L-Galactopyranosyl-(1 → 4)-β-D-xylopyranosyl-(1 → 2)-5-O-trans-feruloyl-L-arabinofuranose is the most complex feruloylated heteroxylan side-chain, that has been reported to date (Saulnier et al., 1995). However, the structures of feruloylated side-chains, especially from maize bran, seem to be more complex than previously thought.

In this paper, we describe the isolation and structural identification of three new complex ferulic acid-containing side-chains from maize bran insoluble fibre. Furthermore, we characterise the first p-coumaric acid-containing side-chain, isolated from maize bran.

2. Results and discussion

2.1. Isolation of feruloylated and p-coumaroylated oligo-/monosaccharides

The isolation procedure of feruloylated and p-coumaroylated saccharides involved mild acidic hydrolysis of maize bran fibre, pre-separation of the hydrolyzate using an Amberlite XAD-2 column, separation by Sephadex LH-20 chromatography, and further separation or purification by GPC on Bio-Gel P-2 and RP-HPLC. The conditions of acidic hydrolysis, optimised for the release of feruloylated heteroxylan side-chains with minimal release of free ferulic acid, have been used as described by Saul-

nier's group (Saulnier et al., 1995). The fraction obtained by eluting the Amberlite XAD-2 column with methanol/water 50/50 was selected to isolate feruloylated and p-coumaroylated saccharides by Sephadex LH-20 chromatography. Four fractions (F1 – F4) were collected from Sephadex LH-20 chromatography using water as eluent (Fig. 1). Fractions F1, F2 and F3 were purified on Bio-Gel P-2 with water. Further separation of the isolated fractions F1 and F4 was performed by semi-preparative RP-HPLC. Two compounds were obtained from F1 (F1a and F1b) (Fig. 2). Separation of F4 gave one major fraction F4b and a minor compound F4a.

2.2. Structural identification of new compounds F1a, F1b, F2 and F4a

Structural identification of two major feruloylated compounds in maize bran hydrolyzates, 5-O-trans-feruloyl-L-arabinofuranose (fraction F4b; compound 4b FA, Fig. 3) and β-D-xylopyranosyl-(1 → 2)-5-O-trans-feruloyl-L-arabinofuranose (fraction F3; compound 3 FAX, Fig. 3) has been described previously (Saulnier et al., 1995). The
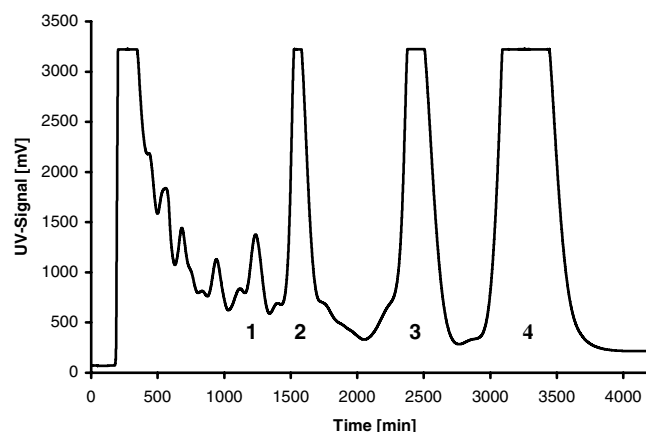

Fig. 1. Sephadex LH-20 chromatogram showing the separation of the acidic maize bran fiber hydrolyzate (MeOH/H$_2$O-fraction after Amberlite XAD-2 separation) using water as eluent (detection at 325 nm).
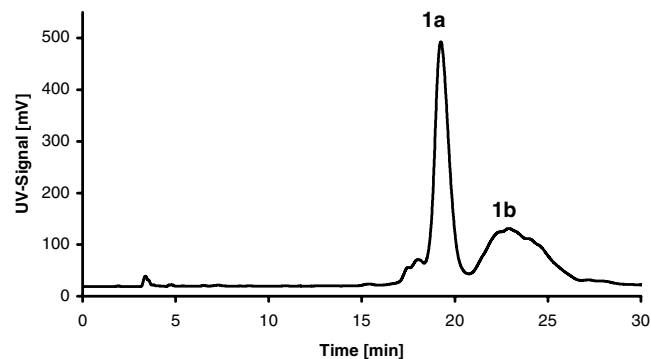

Fig. 2. Semi-preparative RP-HPLC-chromatogram obtained from separation of Sephadex LH-20 fraction F1 under conditions described in the Experimental section.
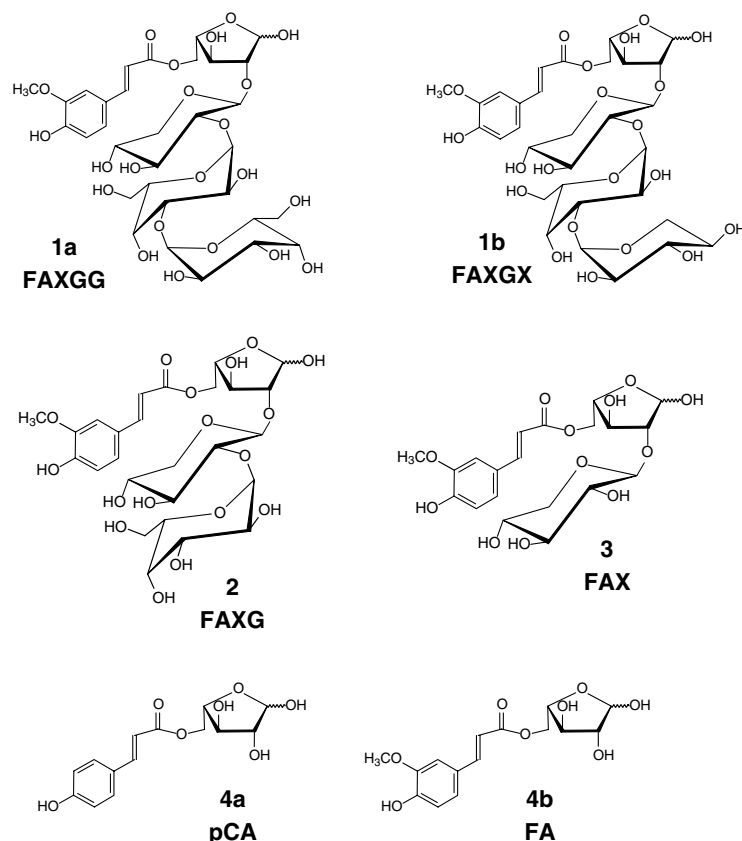
Fig. 3. Structures of the isolated feruloylated mono- and disaccharides **3** and **4b**, which have been previously identified, the feruloylated tetrasaccharides **1a** and **1b**, the feruloylated trisaccharide **2**, and the *p*-coumaroylated monosaccharide **4a**.

structural elucidation of the compounds in F**1a**, F**1b**, F**2** and F**4a** was as follows:

Arabinose was the sole carbohydrate released from F**4a**, determined as its alditol acetate after acidic hydrolysis, reduction and acetylation. F**1a**, F**1b** and F**2** contained arabinose, xylose and galactose in ratios of 1:1:2 in F**1a**, 1:2:1 in F**1b**, and 1:1:1 in F**2**. In all fractions, the absolute configuration of arabinose was L and xylose was D, as determined by chiral GC after acidic hydrolysis and trifluoroacetylation. Analysis of galactose residues from F**1b** and F**2** gave only L-galactose. Conversely, one galactose released from F**1a** was identified as L-galactose, the other as D-galactose (Fig. 4).

Analysis of F**1a** by HPLC-MS gave a sodium adduct ion with m/z 805 $[M+Na]^+$ and a potassium adduct ion with m/z 821 $[M+K]^+$, indicating a molecular mass of 782, corresponding to one ferulic acid, two pentoses (arabinose, xylose) and two hexoses (galactose). Application of high-resolution MS (found $[M+Na]^+$ 805.23765, $C_{32}H_{46}NaO_{22}$ requires M 805.23783) confirmed this structural composition. Positive-ion MS of F**1b** showed m/z 775 $[M+Na]^+$ and m/z 791 $[M+K]^+$, suggesting a molecular mass of 752, corresponding to one ferulic acid, three pentoses (one arabinose, two xyloses) and one hexose (galactose). Again, high-resolution MS (found $[M+Na]^+$ 775.22686, $C_{31}H_{44}NaO_{21}$ requires M 775.22727) confirmed this assumption. The molecular mass of F**2** was determined as

620 (sodium adduct ion with m/z 643 $[M+Na]^+$ and potassium adduct ion with m/z 659 $[M+K]^+$), consistent with a compound built of one ferulic acid, two pentoses (arabinose, xylose) and one hexose (galactose) (high-resolution MS, found $[M+Na]^+$ 643.18589, $C_{26}H_{36}NaO_{17}$ requires M 643.18501). Analysis of F**4a** by HPLC-MS gave the sodium adduct ion with m/z 319 $[M+Na]^+$ and the potassium adduct ion with m/z 335 $[M+K]^+$, indicating a molecular mass of 296, corresponding to one coumaric acid and one pentose (arabinose) (high-resolution MS, found $[M+Na]^+$ 319.07948, $C_{14}H_{16}NaO_7$ requires M 319.07937).

The complete structural elucidation of all isolated compounds is based on one- and two-dimensional NMR experiments. From the phenolic region of the ^1H NMR spectrum of fraction F**4a** the cinnamate moiety was identified as *trans-p*-coumarate (coupling constant $J_{7,8} = 16.0$ Hz). The aromatic region of the ^1H NMR spectrum of fractions F**1a**, F**1b** and F**2** showed typical chemical shifts for *trans*-ferulate esters (Bunzel et al., 2002; Saulnier et al., 1995). Interpretation of the carbohydrate region was more complex. The presence of the α- and β-anomers of the arabinose unit contributes to severe signal overlap. Signals from anomeric protons were used as starting points for the assignment of the proton signals in the H,H-COSY and TOCSY spectra (data presented in Table 1). Carbon chemical shifts of F**1a**, F**2** and F**4a** were taken from the 1D ^{13}C NMR experiment, and of F**1b** from the 2D HSQC
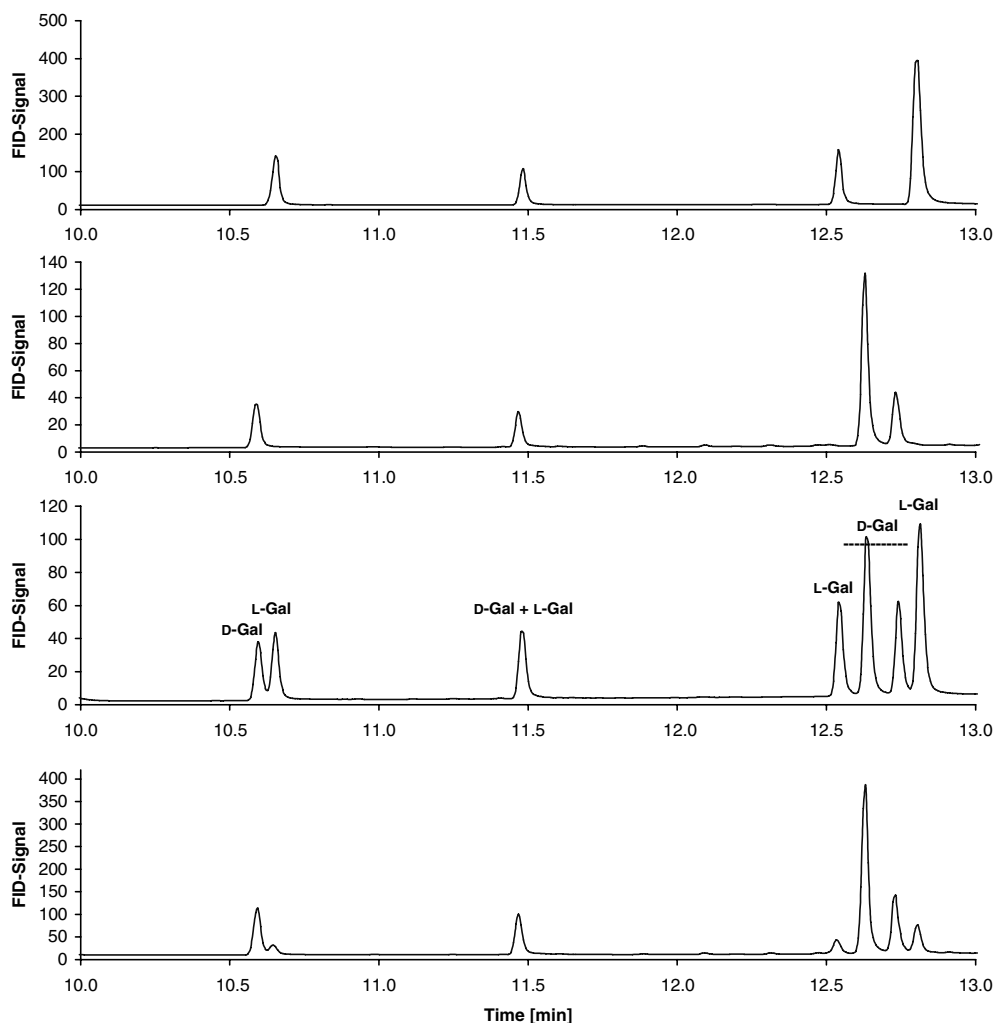
Fig. 4. Gas chromatograms (galactose region) using a chiral capillary column for the separation of trifluoroacetylated monosaccharides. Chromatograms are achieved from the analysis of, from top to bottom, L-galactose, D-galactose, fraction F1a (FAXGG), and coinjection of fraction F1a with D-galactose under conditions pertaining to the procedure described in Section 4.

and HMBC experiments (data presented in Table 2). Assignments of the carbon signals were generally done by interpretation of the HSQC and HMBC spectra. Inter-residue linkages and ring sizes of the carbohydrate units were established by the long-range correlation experiment HMBC. HMBC spectra also allowed the assignment of the position of ferulic acid in the compounds from F1a, F1b and F2, and of p-coumaric acid in F4a. In all compounds the hydroxycinnamic acids acylate the O-5-positions of the arabinosyl units.

Using the described information, the compound in F2 has been identified as α-L-galactopyranosyl-(1 → 2)-β-D-xylopyranosyl-(1 → 2)-5-O-trans-feruloyl-L-arabinofuranose (FAXG) (compound **2** FAXG, Fig. 3). A similar compound, but with a (1 → 4)-linkage between galactose and xylose, has been previously isolated and identified from maize bran (Saulnier et al., 1995). However, the HMBC experiment clearly showed the glycosidic (1 → 2)-linkage between galactopyranose and xylopyranose (Fig. 5). Methylation analysis of F2 confirmed the NMR results and also

demonstrated the (1 → 2)-linkage between galactose and xylose. This compound has not been reported previously.

The compound from F1b has been identified as α-D-xylopyranosyl-(1 → 3)-α-L-galactopyranosyl-(1 → 2)-β-D-xylopyranosyl-(1 → 2)-5-O-trans-feruloyl-L-arabinofuranose (FAXGX) (compound **1b** FAXGX, Fig. 3). The second xylosyl anomeric proton had a coupling constant $J_{1,2} = 3.56$ Hz, implicating the α-form. Usually, the β-anomer, exhibiting a coupling constant around 7-8 Hz, is the predominant glycosidic form of xylose in cereal heteroxylans. The (1 → 2)-linkage between xylopyranose 1 and arabinofuranose, the (1 → 2)-linkage between galactopyranose and xylopyranose 1, and the (1 → 3)-linkage between xylopyranose 2 and galactopyranose were clearly shown in the HMBC spectrum and confirmed by methylation analysis. Fig. 6 shows the HSQC spectrum of the carbohydrate region of fraction F1b. Three signals of protons (Fig. 6) coupling to each other could not be assigned to the identified molecule. Regarding the corresponding carbon signals, two signals (with carbon shifts around 63 ppm) represent

Table 1
^{1}H NMR data of feruloylated and *p*-coumaroylated oligo-/monosaccharides (in D_2O); chemical shifts δ_H in ppm, coupling constants J in Hz

	H1 ($J_{1,2}$)	H2 ($J_{2,3}$)[b]	H3	H4	H5 ($J_{5,6}$)[a]		H6	H7 ($J_{7,8}$)	H8	OMe
					H5eq	H5ax				
FAXG										
α-L-Ara*f*	5.441 (2.14)	4.160	4.170	4.310	4.434	4.306				
β-L-Ara*f*	5.346 (4.26)	4.170	4.300	4.029	4.438	4.256				
β-D-Xyl*p*										
α	4.684 (7.23)	3.502	3.686	3.588	3.961	3.333				
β	4.699 (7.13)	3.621	3.703	3.651	3.976	3.350				
α-L-Gal*p*										
α	5.388 (3.07)	3.816	3.842	3.966	4.195		3.713			
β	5.449 (3.11)	3.837	3.867	3.962	4.350		3.720			
Ferulate										
α		7.095			6.858 (8.1)		7.056	7.535 (15.9)	6.279	3.829
β								7.545 (15.9)	6.281	
FAXGG										
α-L-Ara*f*	5.395 (2.30)	4.112	4.147	4.259	4.385	4.287				
β-L-Ara*f*	5.304 (4.44)	4.142	4.258	3.993	4.398	4.215				
β-D-Xyl*p*										
α	4.641 (7.35)	3.468	3.648	3.537	3.915	3.296				
β	4.643 (7.30)	3.594	3.657	3.597	3.923	3.296				
α-L-Gal*p*1[a]										
α	5.366 (3.76)	3.968	3.894	4.140	4.200		3.670			
β	5.453 (3.67)	3.989	3.943	4.143	4.354		3.677			
α-D-Gal*p*2[a]										
α	5.191 (3.20)	3.756	3.824	3.893	3.966		3.658			
β	5.190 (3.36)	3.766	3.849	3.910	3.989		3.667			
Ferulate										
α		7.106			6.820 (8.0)		7.050	7.536 (15.9)	6.278	3.802
β								7.547 (15.9)	6.282	
FAXGX										
α-L-Ara*f*	5.385 (−)	4.123	4.122	4.271	4.369	4.278				
β-L-Ara*f*	5.306 (4.40)	4.164	4.250	3.993	4.374	4.234				
β-D-Xyl*p*										
α	4.638 (7.47)	3.477	3.641	3.535	3.910	3.287				
β	4.664 (7.42)	3.584	3.650	3.586	3.913	3.287				
α-L-Galp 1										
α	5.401 (3.42)	3.969	3.808	4.049	4.158		3.644			
β	5.460 (3.40)	3.988	3.852	4.041	4.342		3.646			
α-L-Xyl*p*										
α	5.082 (3.56)	3.429	3.637	3.502	3.584	3.545				
β	5.094 (3.56)	3.436	3.643	3.505	3.592	3.552				
Ferulate										
α		7.114			6.824 (8.1)		7.054	7.547 (15.9)	6.286	3.801
β								7.560 (15.8)	6.288	
pCA										
α-L-Ara*f*	5.291 (−)	4.073	4.077	4.340	4.447	4.316				
β-L-Ara*f*	5.325 (4.57)	4.127	4.160	4.043	4.469	4.286				
p-Coumarate										
α		7.547 (8.4)	6.916		6.916 (8.4)		7.547	7.694 (16.0)	6.397	
β								7.701 (16.0)	6.404	

^{a} For the assignment of L- and D-Gal see text.
^{b} Coupling constants for ferulate/*p*-coumarate residue.

secondary carbons; one signal (carbon shift around 73 ppm) represents a primary or tertiary carbon (from phase-sensitive HMQC). Due to their chemical shifts, these signals may also have their origin from carbons/protons at the 5- and 6-positions of galactose, but it was not possible to get correlations to the assigned signals of compound **1b**, neither in the COSY, TOCSY, nor HMBC experiments. However,

the same is true for the proton signals of the assigned galactose protons in 5- and 6-positions in the COSY and TOCSY-experiments where also the link between signals from protons in 4-position and protons in 5-position was missing. It was possible to assign the signals of the carbons at the 5-position in the HMBC spectrum starting from the proton at the 1-position via a three-bond correlation.

Table 2
^{13}C NMR data of feruloylated and *p*-coumaroylated oligo-/monosaccharides (in D$_2$O); chemical shifts δ_C in ppm

	C1	C2	C3	C4	C5	C6	C7	C8	C9	OMe
FAXG										
α-L-Ara*f*	100.27	90.10	75.39	80.08	63.86					
β-L-Ara*f*	95.19	84.01	73.12	78.71	65.11					
β-D-Xyl*p*										
α	102.09	77.52	76.27	69.63	65.17					
β	102.83	76.71	76.52	69.72	65.11					
α-L-Gal*p*										
α	99.03	68.93	69.53	69.58	71.22	61.56				
β	98.82	68.89	69.72	69.72	71.06	61.36				
Ferulate										
α	126.99	111.53	147.96	148.45	115.89	123.84	146.95	114.04	169.47	56.13
β	127.03	111.50					146.85	114.17	169.38	
FAXGG										
α-L-Ara*f*	100.53	90.60	75.57	80.27	64.00					
β-L-Ara*f*	95.37	84.62	73.30	78.96	65.39					
β-D-Xyl*p*										
α	102.59	77.73	76.67	69.84	65.44					
β	103.43	76.48	77.06	69.96	65.52					
α-L-Gal*p* 1[a]										
α	99.24	68.54	77.88	69.65	71.48	61.71				
β	98.85	68.45	77.96	69.72	71.32	61.51				
α-D-Gal*p* 2[a]										
α	101.06	69.38	69.96	69.96	72.10	61.97				
β	101.19	69.38	69.96	69.96	72.14	62.04				
Ferulate										
α	126.88	111.89	148.58	149.56	116.45	124.30	147.33	114.19	169.80	56.48
β	126.95	111.84					147.21	114.32	169.90	
FAXGX										
α-L-Ara*f*	100.7	90.1	75.7	80.5	64.0					
β-L-Ara*f*	95.5	84.1	73.6	79.1	65.2					
β-D-Xyl*p*										
α	102.3	77.2	76.8	69.9	65.5					
β	103.0	76.3	77.1	69.9	65.5					
α-L-Gal*p* 1										
α	99.0	68.4	78.1	69.8	71.5	61.7				
β	98.8	68.4	78.1	69.9	71.3	61.6				
α-L-Xyl*p*										
α	101.3	72.4	73.6	70.0	62.4					
β	101.4	72.4	73.6	70.0	62.4					
Ferulate										
α	126.9	112.0	148.6	149.5	116.5	124.3	147.4	114.2	169.8	56.5
β	127.1	111.8					147.3	114.3	169.9	
pCA										
α-L-Ara*f*	100.93	80.94	75.67	80.42	63.59					
β-L-Ara*f*	95.01	75.61	73.95	78.33	64.66					
p-Coumarate										
α	126.00	130.23	115.66	158.21	115.66	130.23	146.07	113.48	168.95	
β	126.03						146.00	113.60	169.34	

Data of FAXG, FAXGG and pCA are from 1D ^{13}C NMR spectra. Data of FAXGX are from 2D HSQC and HMBC spectra; a 1D ^{13}C NMR spectrum of FAXGX was run but was too weak to deduce peak details.

[a] For assignment of L- and D-Gal see text.

Therefore, the origin of the unassigned signals remains unclear; an impurity remains a possibility.

The compound in **F1a** has been identified as α-D-galactopyranosyl-(1 → 3)-α-L-galactopyranosyl-(1 → 2)-β-D-xylopyranosyl-(1 → 2)-5-*O-trans*-feruloyl-L-arabinofuranose (FAXGG) (compound **1a** FAXGG, Fig. 3). Fig. 7 shows the carbohydrate region of the HSQC spectrum. Again,

the HSQC spectrum of this fraction contained a three-signal impurity, which we could not assign (Fig. 7). The HMBC experiment and results from the methylation analysis indicated (1 → 2)-linkage between xylopyranose and arabinofuranose, (1 → 2)-linkage between galactospyranose 1 and xylopyranose, and (1 → 3)-linkage between galactopyranose 2 and galactopyranose 1. As mentioned before one
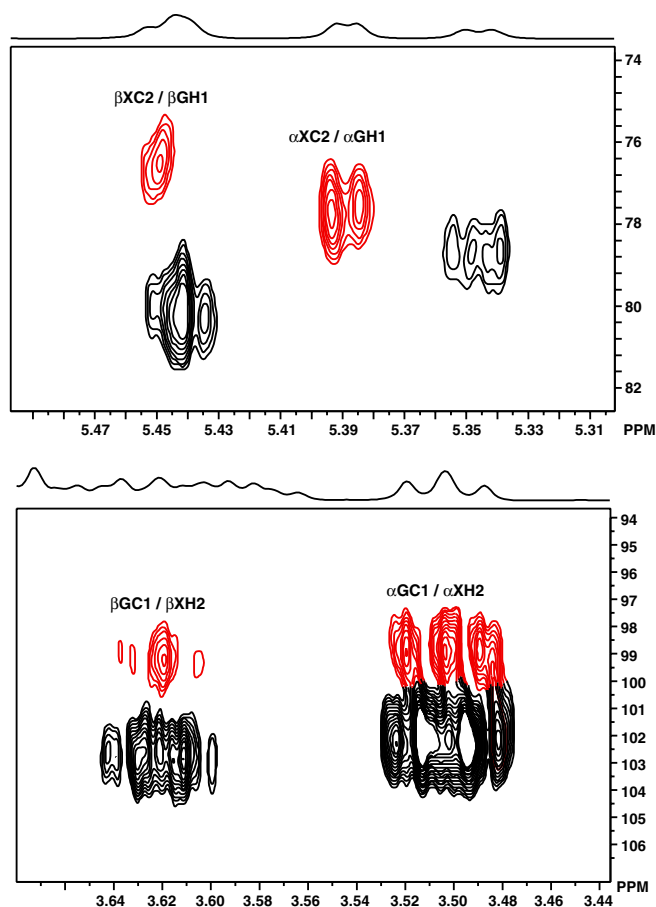
Fig. 5. Slices of the HMBC spectrum of compound 2 (FAXG) showing long-range correlations between galactose H1 and xylose C2 (top), and xylose H2 and galactose C1 (bottom), indicating a Gal (1 → 2) Xyl-linkage.

galactose has an L-configuration and the other a D-configuration (Fig. 4). L-Galactose was proven to be involved in structures 1b and 2. Therefore, we logically assumed that galactose-1, bound to the xylose unit, is L-galactose. As a consequence, galactose-2 must be the one with the D-configuration.

Polysaccharides that contain adjacent D-galactose and L-galactose units have been previously isolated from different families of red seaweed (Nunn et al., 1971; Takano et al., 2003). Adjacent L- and D-galactosyl residues were also identified in the side-chains of jojoba seed xyloglucan oligosaccharides (Hantus et al., 1997). This report broadens the quite unusual finding of neighboring L-galactose- and D-galactose in polysaccharides to heteroxylans. In addition, FAXGG and FAXGX are the most complex feruloylated heteroxylan side-chains that have been isolated to date.

Finally, the compound in F4a has been identified as 5-O-trans-p-coumaroyl-L-arabinofuranose. p-Coumaroylated heteroxylan side-chains have not been isolated from cell walls of maize bran and cereal grains in general before. A p-coumaroylated arabinoxylan trisaccharide and tetrasaccharide were isolated from cell walls of barley straw (Ishii,

1997; Mueller-Harvey et al., 1986) and bamboo shoot (Ishii et al., 1990; Ishii, 1996, 1997).

3. Conclusions

The isolation of three novel feruloylated saccharides from maize bran insoluble fibre shows that the structures of feruloylated side-chains from heteroxylans are more complex than expected. The presence of α-xylose in the feruloylated tetrasaccharide (FAXGX) was surprising, because the β-anomer of xylose usually dominates in cereal heteroxylans. Also the second feruloylated tetrasaccharide (FAXGG) is unusual because it contains neighbouring L-galactose and D-galactose units. This linkage between two differentially configured galactose units has been demonstrated only for a few polysaccharides to date. The isolated trisaccharide has been identified as α-L-galactopyranosyl-(1 → 2)-β-D-xylopyranosyl-(1 → 2)-5-O-trans-feruloyl-L-arabinofuranose (FAXG). From former studies a (1 → 4)-linkage between galactose and xylose was expected, but the structure shown here with a (1 → 2)-linkage was unambiguously demonstrated. The isolation of a p-coumaric acid-containing side-chain from maize bran suggests the possibility that cereal grain polysaccharides are possibly also cross-linked via p-coumarates to other polysaccharides, lignin and proteins. Complex feruloylated heteroxylan side-chains may limit enzymatic degradation of fibre.

4. Experimental

4.1. Plant materials

Maize bran (Zea mays L.) was kindly provided by Hammermühle Maismühle GmbH (Kirrweiler, Germany).

4.2. General

Heat-stable α-amylase Termamyl 120 L (EC 3.2.1.1, from Bacillus licheniformis, 120 KNU/g) was from Novo Nordisk (Bagsvaerd, Denmark). Amberlite XAD-2 was obtained from Serva (Heidelberg, Germany), Bio-Gel P-2 from Bio-Rad Laboratories (Hercules, CA, USA), and Sephadex LH-20 was from Pharmacia Biotech (Freiburg, Germany). Acetonitrile (HPLC grade) was from Acros (Geel, Belgium), and deuterium oxide from Sigma (Milwaukee, WI, USA). All other chemicals and solvents were purchased from Merck (Darmstadt, Germany).

4.3. Preparation of insoluble maize fibre

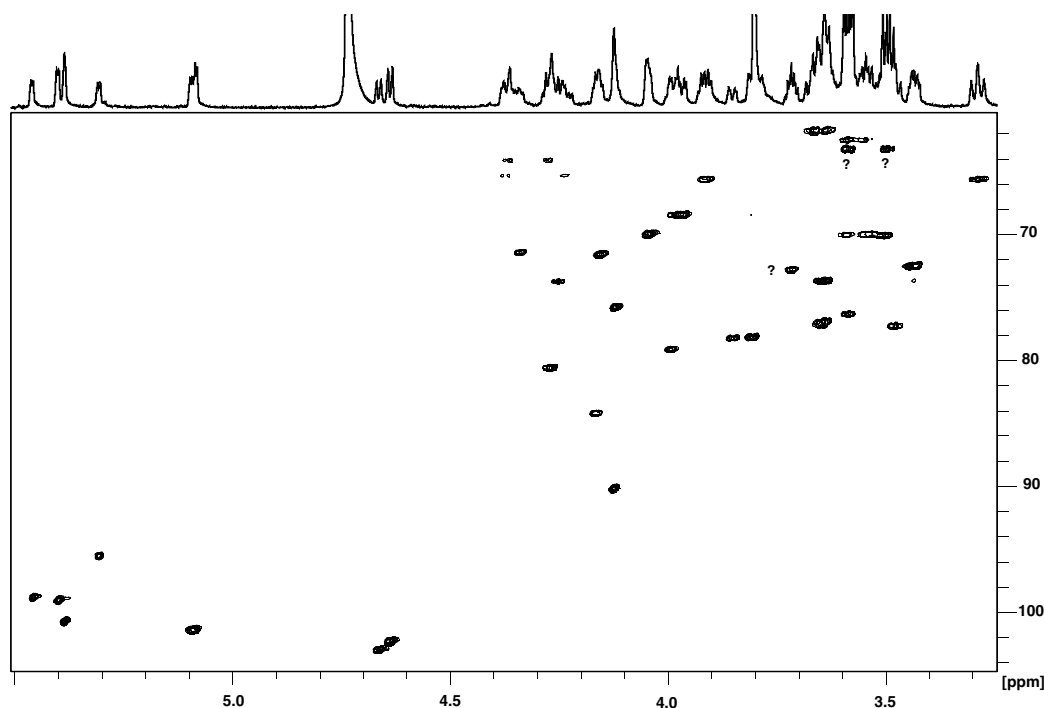n-Hexane defatted maize bran (20 g, milled to a particle size smaller than 0.5 mm) was suspended in phosphate

Fig. 6. Carbohydrate region of the HSQC spectrum of compound **1b** (FAXGX). Correlation peaks indicated with a question mark were not assigned (see text).
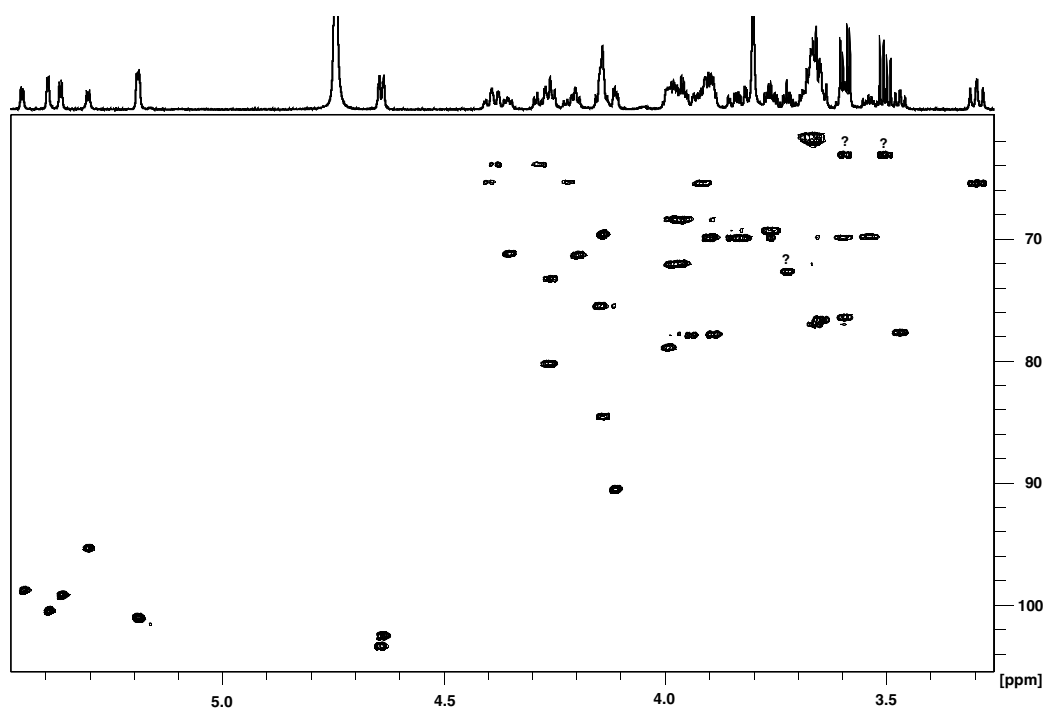

Fig. 7. Carbohydrate region of the HSQC spectrum of compound **1a** (FAXGG). Correlation peaks indicated with a question mark were not assigned (see text).

buffer (pH 6.0, 0.08 M, 300 ml), and heat-stable α-amylase (1.5 ml) was added. Beakers were placed in a boiling water bath for 40 min and shaken every 5 min. After cooling to room temperature, the suspension was centrifuged, and

the residue was washed twice with hot water (70 °C), 95% (v/v) ethanol and acetone, and was finally dried at 40 °C overnight in a vacuum oven. This procedure was performed several times.

4.4. Chemical degradation of insoluble fibre

Chemical degradation of insoluble fibre was carried out as described previously (Allerdings et al., 2005; Saulnier et al., 1995). Insoluble fibre (55 g) was treated with 50 mM trifluoroacetic acid (TFA, 1.2 l) under reflux for 3 h at 100 °C. After centrifugation the supernatant was filtered and evaporated at 40 °C under vacuum to 200 ml. The solution was adjusted to pH 5.0 using 0.1 M NaOH and centrifuged again. Finally, the solution was concentrated to 100 ml.

4.5. Isolation of feruloylated and p-coumaroylated oligo-/monosaccharides

The hydrolysate was applied to a column (40 × 5 cm) of Amberlite XAD-2. Elution was carried out with H_2O (1.5 l), $MeOH/H_2O$ 50/50 (2 l) and MeOH (1.5 l). The $MeOH/H_2O$ fraction was concentrated to 25.5 ml and applied to a column (100 × 3.2 cm) of Sephadex LH-20. Elution was performed with water (flow rate: 1 ml min^{-1}; L-6000 pump, Merck/Hitachi, Darmstadt, Germany) for 70 h. The absorbance of the eluent was monitored continuously at 325 nm with an UV-detector equipped with a preparative flow cell (L-7400, Merck/Hitachi, Darmstadt, Germany). Fractions were collected every 18 min.

The isolated fractions F1, F2, and F3 (Fig. 1) were concentrated to 5 ml and applied to a column (100 × 2.5 cm) of Bio-Gel P-2 for further purification. Elution was performed with water (flow rate: 0.5 ml min^{-1}; L-6000 pump, Merck/Hitachi, Darmstadt, Germany). The absorbance of the eluent was monitored continuously at 325 nm with an UV-detector equipped with a preparative flow cell (L-7400, Merck/Hitachi, Darmstadt, Germany). Fractions were collected every 12 min.

Further separation of the isolated fraction F4 (Fig. 1) and final separation of fraction F1 were performed by semi-preparative HPLC (L-6200 Intelligent pump, Merck/Hitachi, Darmstadt, Germany) using a Nucleosil 100-5 C18 HD column (250 × 10 mm, 5μm, Macherey-Nagel, Düren, Germany). Elution was carried out at room temperature and a flow rate of 4 ml min^{-1} was maintained. An acetonitrile/water gradient was used: initially acetonitrile/water 12/88, held isocratically for 25 min, linear over 5 min to acetonitrile/water 40/60, held isocratically for 10 min, followed by a rinsing and equilibration step. The injection volume was 50 μl using a 100 μl injection loop. Phenolic compounds were detected at 325 nm using an UV-detector equipped with a preparative flow cell (L-7400, Merck/Hitachi, Darmstadt, Germany).

Using the described methodologies, about 100 mg of compound 2, 10 mg each of compounds 1a and 1b, and about 1 mg of compound 4a were isolated out of 100 g insoluble maize fibre. However, since the separation procedures for each compound differ in detail, the amounts are not fully comparable and can only approximate their frequencies of occurrence.

4.6. Identification of feruloylated and p-coumaroylated oligo-/monosaccharides

Molecular weights of the isolated feruloylated and p-coumaroylated oligo-/monosaccharides were determined using ESI-HPLC-MS working in the positive-ion mode (HP Series 1100: autosampler G1313A, bin pump G1312A, degasser G1322A, mass spectrometer G1946A (ion-source: atmospheric pressure electro-spray ionization), Hewlett–Packard, Waldbronn, Germany). Elution was carried out using a Nucleosil 100-5 C18 HD column (250 × 4 mm, 5 μm, Macherey-Nagel, Düren, Germany) and a gradient of ammonium formate buffer (pH 3) and acetonitrile was used as eluent: initially acetonitrile/ammonium formate 12/88, linear over 30 min to acetonitrile/ammonium formate 15/85, linear over 2 min to acetonitrile/ammonium formate 50/50, held isocratically for 2 min. The injection volume was 20 μl, the column temperature 40 °C, and the flow rate was maintained at 1 ml min^{-1}. High-resolution ESI-MS was performed on a MAT95XL (ThermoFinnigan, Bremen, Germany) instrument, working in the positive-ion mode. The sample was injected using a syringe pump.

Neutral carbohydrate compounds were determined as alditol acetates after acidic hydrolysis (2 M TFA, 120 °C, 60 min) by GC-FID (Hewlett–Packard 5890 Series II, Waldbronn, Germany). Reduction and acetylation was carried out as described elsewhere (Blakeney et al., 1983). Separation of the resulting alditol acetates was performed as described previously (Allerdings et al., 2005).

Methylation analysis of feruloylated oligosaccharides was carried out as described by Nunes and Coimbra (Nunes and Coimbra, 2001) but with minor modifications. Samples (1–2 mg) were dissolved in dry DMSO (2 ml). NaOH pellets (100 mg) were freshly powdered under nitrogen and added to the solution. Each sample was sonicated for 90 min and stood for another 90 min at room temperature. Methyl iodide (1 ml) was added to the ice-cold solution and allowed to react for 30 min in an ultrasonic bath and another 30 min at room temperature without sonication. Water (3 ml) was added, and the methylated material was extracted into chloroform (1 ml). The organic layer was washed five times with water (5 ml) and evaporated under a stream of nitrogen at 40 °C. Samples were hydrolysed with 2 M TFA (1 ml) at 121 °C for 1 h and dried under a stream of nitrogen at 40 °C. The partially methylated sugars were dissolved in a freshly prepared solution of 20 mg $NaBD_4$ in 0.3 ml of 2 M aqueous NH_3. The mixture was allowed to react at room temperature for 1 h, and the reaction was stopped by adding glacial acetic acid (0.1 ml). Partially methylated alditols were acetylated by adding 1-methylimidazole (0.45 ml) and acetic anhydride (3 ml). Following a reaction time of 30 min, water (3 ml) was added to decompose the residual acetic anhydride. Partially methylated alditol acetates were extracted into dichloromethane (2 ml) and determined by GC-MS (HP 5890 Series

II GC, HP 5972 Series Mass Selective Detector, Hewlett-Packard, Waldbronn, Germany) using a 0.25 mm × 30 m DB-5MS capillary column (0.25 μm film thickness) (J&W Scientific, Folsom, CA, USA) capillary column. GC conditions were as follows: initial column temperature was 45 °C, held for 1 min, ramped at 45 °C min^{-1} to 140 °C, held for 1 min, ramped at 1 °C min^{-1} to 180 °C, held for 5 min; splitless injection (splitless time 0.75 min, injector temperature 210 °C), mass spectrometric detection (ionisation energy 70 eV). He (1.08 ml min^{-1}) was used as carrier gas.

The absolute configuration of monosaccharides from isolated fractions was determined by chiral GC analysis using a SatoChrom GC and a 0.25 mm × 25 m Hydrodex-β-6-TBDM chiral capillary column (Macherey-Nagel, Düren, Germany). Samples were hydrolysed with 2 M trifluoroacetic acid (1 ml) at 121 °C for 1 h and dried under a stream of nitrogen at 40 °C. The residues were suspended in dichloromethane (2 ml), and trifluoroacetic anhydride was added (100 μl). The mixtures were allowed to react at room temperature overnight and dried under a stream of nitrogen at 40 °C. The sugar derivatives were separated using the following temperature program: initial column temperature was 100 °C, held for 5 min, ramped at 10 °C min^{-1} to 220 °C, held for 10 min. Split injection (injector temperature 250 °C), flame ionisation detection (detector temperature 250 °C) and hydrogen as carrier gas were used.

1D and 2D NMR-spectra were acquired on Bruker DMX-750 and/or DRX-500 instruments (Bruker, Rheinstetten, Germany). Samples (3–15 mg) were dissolved in 0.5–0.7 ml D$_2$O; acetone (0.5 μl) was used as reference (δ_H 2.22, δ_C 30.89) (Gottlieb et al., 1997). Experiments used (^{1}H, ^{13}C, H, H-COSY, TOCSY, HSQC/HMQC, HMBC) were standard Bruker implementations.

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